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Interactions between aged biochar, fresh low molecular weight carbon and soil organic carbon after 3.5 years soil-biochar incubations

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ABSTRACT

Biochar has been considered a stable additive for long-term soil carbon (C) storage and climate change mitigation. However, our understanding of the long-term interactions between aged biochar, soil organic C (SOC), and fresh low molecular weight C compounds (LMW-C) is still limited. In addition to the changes in the chemical properties of biochar and the SOC after aging, biochar stability and C sequestration potentials might be affected by the LMW-C availability. After 3.5 years of incubation, aged soils and aged soil-biochar mixtures (4 atom‰ ^{13}C enriched grass-derived biochar, 10% addition rate) of two different soil types were used in this study, with or without the addition of LMW-C. The ^{13}C natural abundance of the LMW-C was manipulated by mixing cane sucrose (C4) and beet sugar (C3) to make it different from or equal to the native SOC isotopic signature of the two soil types, which allowed the partitioning of the LMW-C and biochar C from the SOC in non-biochar and biochar addition treatments, respectively. We traced the ^{13}C in the cumulative CO_2, microbial biomass C (MBC), and dissolved organic C (DOC) at the beginning and the end of the 28 days of incubation. Results indicated that this aged biochar was not as effective for SOC protection as it was when fresh. Aged biochar caused significant increases in the native SOC-derived CO_2 compared to non-biochar treatment during the 3.5 years of aging. Within the 28 days of incubation, aged biochar not only promoted the total CO_2 emission but also did not limit the priming effect of LMW-C on native SOC mineralization. SOC-derived DOC in the aged soil-biochar mixture was significantly higher than that in the aged soil, which further increased the SOC loss. A decline in the SOC stability with aged biochar might be associated with the attenuated sorption of SOC on aged biochar, the decline in bulk density with the high biochar addition rate, and a modified microbial community. The priming of aged SOC mineralization with the addition of LMW-C might be associated with the enhanced microbial N demand in aged soils and soil-biochar mixtures. The effect of aged biochar on the soil DOC and the effect of LMW-C on the aged biochar and SOC stabilities should be further investigated in field conditions, under continuous fresh C inputs, to better evaluate the effects of biochar on long-term SOC storage.

1. Introduction

Soil organic C (SOC) plays a key role in maintaining soil functions and quality, supporting the productivity of agroecosystems (Lal, 2010). Although soils (to a depth of 1 m) contain almost 50% of the terrestrial C pool, previous research has reported that land use changes and human activities have reduced C stocks by ~133 Pg C in agricultural lands (Sanderman et al., 2017). Biochar was considered to be a stable additive for long-term soil C storage (Singh et al., 2015). Biochar can decrease the turnover rates of both existing SOC and new plant root-derived low molecular weight C compounds (LMW-C); therefore, biochar may facilitate the negative priming of SOC mineralization (Whitman et al., 2014; Keith et al., 2015; Weng et al., 2015), in particular in soils with low SOCs (Stewart et al., 2013). However, the SOC sequestration potential of biochar and its interaction with native SOC and fresh LMW-C may change after aging in soil, and related information is lacking.

Previous studies suggested that the addition of LMW-C or biochar could promote microbial activity or change microbial communities to enhance SOC mineralization and influence SOC stability through co-

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metabolism (Farrell et al., 2013; Zhang et al., 2014; Jiang et al., 2016a). Maestrini et al. (2015) found that fresh biochar showed a positive priming effect on the native SOC in the first 20 days. Fresh C input (such as LMW-C) could even significantly decrease the stability of aged SOC in deep soil (Fontaine et al., 2007). The priming effects of the addition of biochar on SOC mineralization have been reported in different experiments (Conant et al., 2011; Zimmermann et al., 2012; Lu et al., 2014; Weng et al., 2015; Jiang et al., 2016b), but generally were limited to experiments with fresh biochar. Fresh biochar contains some labile matter that can be utilized by soil microbes (Dong et al., 2017); therefore, native SOC mineralization can be promoted (positive priming) by the addition of fresh biochar. It was believed that most of the labile biochar material was leached or dissolved during aging (Dong et al., 2017), and a negative priming effect of the addition of biochar on SOC mineralization would appear at later stages (> 200 days) (Maestrini et al., 2015). However, aged biochar also contains more O-rich functional groups compared to fresh biochar, which can lead to significant attenuation of the sorption capacity of SOC on aged biochar (Luo et al., 2017). These changes in functional groups may enhance biochar solubility and decrease the SOC sequestration capability of biochar. Therefore, it is still necessary to study SOC mineralization under aged biochar in long-term experiments.

The formation of mineral-organic associations has been demonstrated to be a key in controlling SOC protection and stabilization (Kleber et al., 2015). It has been proposed that biochar can promote mineral-organic interactions through adsorption and ligand exchange (Joseph et al., 2010; Singh et al., 2012), but this facilitation effect may weaken with the attenuation of the sorption capacity on aged biochar during incubation. Furthermore, LMW-C have been found to accelerate the transformation and turnover of mineral-organic associations (Kleber et al., 2015), which may stimulate SOC loss. The priming of SOC mineralization may also depend on biochar and LMW-C interactions, with the magnitude and direction of priming effects depending on the duration of the experiment and degree of biochar aging (Maestrini et al., 2015; Wang et al., 2016). It was reported that the addition of LMW-C suppressed SOC mineralization in the presence of aged biochar, which could be attributed to the preferential use of LMW-C over the more recalcitrant C in aged biochar and aged SOC (Blagodatskaya and Kuzyakov, 2008; Wang et al., 2016), which was in contrast to the co-metabolism mechanism. Overall, the mechanisms of SOC retention and loss within soil systems, in which LMW-C interacts with aged SOC and biochar, remain unknown. Uncertainty in the interactions between SOC, fresh LMW-C, and aged biochar will limit our ability to predict effects of biochar additives on the soil long-term C sequestration in the future.

To understand the interactions between SOC, fresh LMW-C, and aged biochar, we added LMW-C to soil and soil-biochar mixtures aged 3.5 years, with two levels of LMW-C addition, and we repeated the experiment we had conducted using fresh biochar (Jiang et al., 2016b). We traced the losses of C (CO_2 and DOC) from the decomposition of SOC, LMW-C, and aged biochar. Isotope-manipulated LMW-C (different or the same $\delta^{13}C$ as native SOC) and ^{13}C-labelled biochar were used to partition the C losses from the SOC and LMW-C (non-biochar treatment) as well as from aged biochar and SOC + LMW-C (biochar addition treatment). In the short-term experiment, the addition of fresh biochar significantly decreased the SOC mineralization, and this negative effect was not influenced by the addition of N or LMW-C (Jiang et al., 2016b). However, it was uncertain whether the negative effect of biochar on SOC mineralization would change after the aging of biochar. We hypothesized as follows: 1) the addition of LMW-C would increase the aged SOC and aged biochar decomposition due to the priming effect; 2) aged biochar would depress the LMW-C stimulation of SOC mineralization; and 3) the soil C sequestration capability of aged biochar would not be affected by the addition of fresh LMW-C.

2. Materials and methods

2.1. Aged soil-biochar mixtures

The aged soil (without biochar) and aged soil-biochar mixtures had been incubated for 3.5 years since October 10, 2013. Biochar materials and soil samples were obtained from a previous short-term biochar incubation experiment (Jiang et al., 2016b). ^{13}C-labelled (4.348 ^{13}C atom‰) bluestem (*Andropogon gerardii*) was used to produce ^{13}C-labelled biochar. The biochar was produced by slow pyrolysis at 450 °C for 4 h and used in the incubation of the soil-biochar mixture. Detailed information about the ^{13}C-labelled feedstock and biochar production was described in a previous study (Soong and Cotrufo, 2015). Soil samples were collected from two grasslands (0–20 cm): Briggsdale, Colorado, USA, and Woodward, Oklahoma, USA. Detailed information about the sampling sites and initial soil properties can be found in Jiang et al. (2016b). After collection, soil samples from different sampling locations were first thoroughly mixed, and then, large roots and stones were removed. Prior to use, soil samples were sieved (< 2 mm), homogenized, and air-dried. The pressure-transducer method was used to determine the soil inorganic C (Sherrod et al., 2002), but no detectable inorganic C was found in either of the two soils.

The volatile matter, ash, and biochar-C, H, O, and N contents were measured by Hazen Research, Inc. (Golden, USA). The Brunauer-Emmett-Teller (BET) method was used to determine the surface area of biochar (Nakayama et al., 1996). The physical and chemical properties of the ^{13}C-labelled biochar are listed in Table 1.

The soil-biochar aging incubation consisted of two soils (Colorado and Oklahoma) and two biochar addition treatments (0 and 10% biochar addition by weight), with 3 replicate units for each combination. The sample without the addition of biochar included a 100 mL plastic cup with 60 g of soil only. The biochar treatment sample included a 100 mL plastic cup containing 6 g of biochar and 60 g of soil. The Wilcox method was used to measure the water holding capacity (WHC) of each treatment prior to incubation (Colman, 1947). All units were incubated in the dark at 25 °C and maintained at 60% WHCs for 3.5 years. Within the 3.5 years incubation, no fresh C input was added to these samples.

After 3.5 years of aging, aged soil and soil-biochar mixtures were used for the LMW-C addition experiment. The SOC, soil N (SN), C:N, and $\delta^{13}C$ of aged soil and soil-biochar mixtures were measured prior to the LMW-C experiment (Table 2). The native SOC, native SN, and residual biochar C of aged soil and soil-biochar mixtures were calculated using the stable isotope mixing model (Balesdent and Balabane, 1992) described below. Then, the loss rates (g kg^{-1} yr^{-1}) of native SOC, native SN and biochar C during the 3.5 years of aging were calculated.

Table 1
Physical and chemical properties of the ^{13}C-labelled biochar obtained from the slow-pyrolysis of bluestem grass.

C	N	O	H	Volatile matter	Ash	C:N	O:C	H:C	pH (1:5)	$\delta^{13}C$	$\delta^{15}N$	Surface area
(%)												(m^2 g^{-1})
50.03	1.52	39.94	1.97	17.68	6.31	32.91	0.80	0.039	9.65	3104.70	19,378.98	6.34

Table 2
Properties of aged soil and soil-biochar mixtures after 3.5 years of incubation.

Soil site	Biochar addition treatments (%)	Organic C (%)	Total N (%)	C:N	$\delta^{13}C$	$\delta^{15}N$	pH (soil:water = 1: 2.5)
Colorado	0	0.62 ± 0.03	0.084 ± 0.005	7.38 ± 0.06	−22.44 ± 0.08	7.04 ± 0.92	7.76 ± 0.08
	10	5.50 ± 0.28	0.25 ± 0.01	22.16 ± 0.32	2753.83 ± 21.23	12,667.18 ± 265.55	7.54 ± 0.09
Oklahoma	0	0.58 ± 0.01	0.066 ± 0.001	8.80 ± 0.16	−20.57 ± 0.24	5.36 ± 0.31	7.68 ± 0.17
	10	5.70 ± 0.23	0.24 ± 0.01	23.84 ± 0.34	2744.56 ± 11.79	13,683.93 ± 104.89	7.54 ± 0.09

2.2. LMW-C addition experiment

Two 30 g samples of moist soil were weighed out from each aged soil or aged soil-biochar mixture replicate unit and transferred into two 100 mL plastic cups. One cup was used for the addition of LMW-C, while the other one was used as a control without the addition of LMW-C. In order to partition the CO_2, dissolved organic C (DOC) and microbial biomass C (MBC) between C derived from SOC + LMW-C and biochar in biochar addition treatments, we used the same approach we had successfully used in a previous study (Jiang et al., 2016b). Beet sucrose (C3, $\delta^{13}C = -24.43$) and cane sucrose (C4, $\delta^{13}C = -12.31$), with same C concentration (43.99%) and chemical structure, were mixed to obtain two different LMW-C samples. We mixed 8.36 g of beet sugar and 1.64 g of cane sucrose to produce a LMW-C with the same $\delta^{13}C$ as Colorado soil ($\delta^{13}C = -22.44$, Table 2), while 6.81 g of beet sugar and 3.19 g of cane sucrose were mixed to produce a LMW-C with the same $\delta^{13}C$ as Oklahoma soil ($\delta^{13}C = -20.57$, Table 2). In order to partition the CO_2, DOC, and MBC between C derived from the SOC and LMW-C in treatments without the addition of biochar, cane sucrose ($\delta^{13}C = -12.31$) was used as the LMW-C.

The LMW-C addition experiment consisted of two soil types (Colorado and Oklahoma), two biochar addition treatments (0 and 10% biochar addition by weight), and two LMW-C addition treatments (0 and 0.10 mg C g^{-1} soil), with 3 replicates of each treatment combination. The LMW-C was added at 7 day intervals throughout the experiment to mimic a field-like continuous fresh C availability. LMW-C was added at 25 μg C g^{-1} dry weight soil for each addition event, which was similar to previous studies (Strickland et al., 2012; Jiang et al., 2016b). The LMW-C was dissolved in a small amount of deionized water and then uniformly added into soil samples. The amounts and concentrations of the LMW-C solutions added were determined according to the water loss of the past 7 day interval. All cups were separately placed in 1 L airtight jars, and all soil samples were maintained at 60% WHC throughout the entire experiment. WHCs of each treatment (with and without biochar addition) were determined for the two soils (Colorado and Oklahoma) using the Wilcox method (Colman, 1947). WHCs of soils with and without biochar addition were 0.236 and 0.295 g H_2O g^{-1} soil, respectively, for Colorado soil; and 0.225 and 0.272 g H_2O g^{-1} soil, respectively, for Oklahoma soil. The LMW-C addition experiment was conducted for 28 days with 4 LMW-C addition events. All airtight jars were incubated in the dark at 25 °C, and the jar headspaces were periodically aerated during the experiment, as described below. After 28 days of incubation, the soil pH, MBC, DOC, dissolved N (DN), MBC-^{13}C, and DOC-^{13}C were analyzed according to the methods described below.

2.3. Soil respiration and $\delta^{13}C$-CO_2

Throughout the experiment, gas samples were collected from the jar headspace to detect the accumulated CO_2 and $\delta^{13}C$-CO_2 concentrations. Briefly, a 2 mL gas sample was collected using a syringe after thoroughly mixing the air in the jar headspace, and then, the accumulated CO_2 concentration was determined using an infrared gas analyzer (IRGA, Lincoln, NE). For $\delta^{13}C$-CO_2 analysis, 20 mL of air was sampled and immediately injected into a vial (12 mL). A Picarro G2131-I Cavity Ring Down Spectrometer (CRDS) was used to measure the $\delta^{13}C$-CO_2. After gas sample collection, all jars were opened, aerated, and closed again. In order to obtain the CO_2 concentration at time 0, the CO_2 concentration in the headspace of each jar were measured immediately as described above. A CO_2 standard with a known concentration was used to calibrate the IRGA at the start of each run and a CO_2 standard with known concentration and $\delta^{13}C$ value was used to calibrate the Picarro at the start of each run.

2.4. Soil analysis

After 28 days, soil samples of all treatments were used for analysis of the soil properties. Soil samples were homogenized by mixing thoroughly with a spatula and subsamples were weighted out for different analysis. The soil pH was determined in a 1:2.5 soil (g)/water (mL) solution. For DOC and DN analysis, 10 g of sample was extracted with 0.5 M K_2SO_4 solution (50 mL) and shaken for 2 h (200 rpm). Solutions were filtered using 40# Whatman filter paper, and then, a Shimadzu TOC analyzer (TOC-L, Japan) was used to measure the DN and DOC of solutions. The MBC was analyzed by the fumigation-extraction method (Vance et al., 1987). Ten grams of moist sample was fumigated in a sealed chamber with alcohol free chloroform under vacuum for 24 h. After fumigation, the fumigated and a non-fumigated (equivalent amount) samples were extracted using 0.5 M K_2SO_4 solution (50 mL) and shaken for 2 h (200 rpm). The extract was filtered using 40# Whatman filter paper, and then, the DOC of the extract was measured using a Shimadzu TOC analyzer (TOC-L, Japan). The MBC was calculated as the DOC difference between non-fumigated and fumigated samples. The extract was freeze-dried in a lyophilizer (Labconco, Missouri, USA), and then, the $\delta^{13}C$ of freeze-dried extract was determined according to the method of Murage and Voroney (2007). An elemental analyzer (Milano, IT) connected with a VG Isochrom continuous flow IRMS (Isoprime, UK) was used to determine the %C, %N, and $\delta^{13}C$ of soil, biochar, and LMW-C samples.

2.5. Data analysis

A stable isotope mixing model (Balesdent and Balabane, 1992) was used to partition CO_2, MBC, DOC, native SOC or SN between different C or N sources, using Eq. (1).

$$f = \frac{\delta_s - \delta_c}{\delta - \delta_c} \quad (1)$$

where f is the fraction of native SOC, SN, LMW-C or biochar C in the CO_2, MBC or DOC; δ_s is the $\delta^{13}C$ or $\delta^{15}N$ of the measured sample; δ is the $\delta^{13}C$ or $\delta^{15}N$ of the LMW-C, biochar C, or biochar N; and δ_c is the $\delta^{13}C$ or $\delta^{15}N$ of the "only soil" treatment.

The MBC-$\delta^{13}C$ was calculated using Eq. (2) as described below:

$$\delta^{13}C = \frac{(\delta^{13}C_{fum} \times C_{fum}) - (\delta^{13}C_{nfum} \times C_{nfum})}{(C_{fum} - C_{nfum})} \quad (2)$$

where C_{nfum} and C_{fum} are the C contents of non-fumigated and fumigated extracts, respectively, and $\delta^{13}C_{nfum}$ and $\delta^{13}C_{fum}$ are the $\delta^{13}C$ of non-fumigated and fumigated extracts, respectively.

SPSS 18.0 software (SPSS Inc., USA) was used for statistical analysis. Repeated ANOVA measurements were used to test the effects of the soil types, biochar, and LMW-C as well as their interactions on CO_2 emission

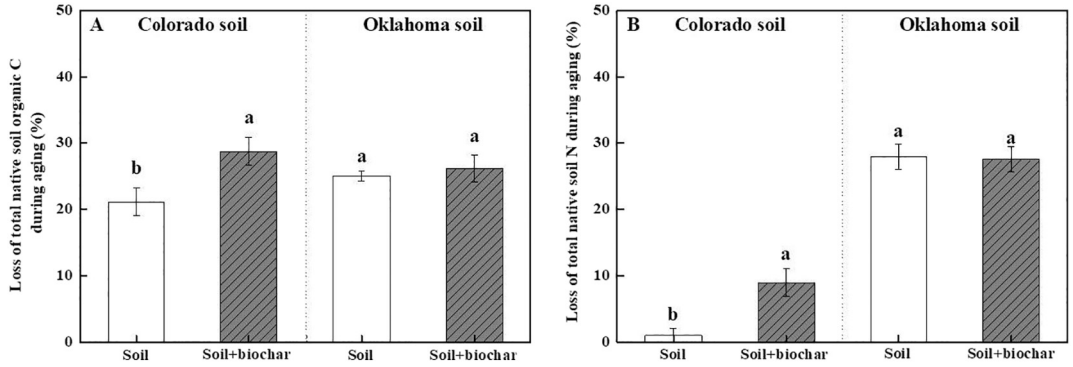

Fig. 1. Loss percentages of A) total native soil organic C and B) total native soil nitrogen during aging with or without the addition of biochar for the Colorado and Oklahoma soils, respectively. Error bars show the standard error (n = 3). Different lowercase letters indicate significant differences within each parameter among different treatments.

over time, SOC-derived CO_2 over time, and biochar-derived CO_2 over time at the 0.05 level. ANOVA analysis was also used to test the effects of soil types, biochar, and LMW-C as well as their interactions with the cumulative SOC-derived CO_2, cumulative biochar-derived CO_2, SOC-derived MBC and DOC, biochar-derived MBC and DOC, DN, and soil pH after the LMW-C experiment at the 0.05 level with the soil type, biochar, and LMW-C as main factors.

3. Results

3.1. Properties of aged soil and soil-biochar mixtures

Regardless of the soil type, there was no significant difference in the pH between aged soil and the soil-biochar mixture, with values ranging from 7.54 to 7.76 (Table 2). The C:N ratio of the aged soil-biochar mixture was three times higher than that of the aged soil. Interestingly, the native SOC of Colorado soil decreased 21.17% and 28.74% for aged soil and soil-biochar mixture, respectively (Fig. 1; $p < 0.05$). Meanwhile, the native SN of Colorado soil decreased 1.06% and 9.01% for aged soil and soil-biochar mixture, respectively (Fig. 1). For the Oklahoma soil, there is no difference in the total native SOC and SN between the aged soil-biochar mixture and aged soil (Fig. 1). Additionally, the 3.5 years incubation caused 4.27% and 1.29% of biochar C loss in the Colorado and Oklahoma soils, respectively.

3.2. Soil respiration

The cumulative CO_2 emission was significantly affected by the addition of biochar and LMW-C addition (Fig. 2; Table 4; $p < 0.001$), but no synergistic effect was found ($p > 0.05$). For the Colorado soil, the cumulative CO_2 emission was increased 66.97%, 122.76%, and 210.88%, respectively, by biochar, LMW-C, and biochar + LMW-C treatments compared to the "only soil" treatment. Similarly in the Oklahoma soil, the biochar, LMW-C, and biochar + LMW-C treatments increased the cumulative CO_2 emission by 33.31%, 119.39%, and 185.03%, respectively, compared to "only soil" treatment (Fig. 2).

The increased CO_2 emission of the soil + LMW-C treatment was mainly derived from the SOC rather than from the added LMW-C (Fig. 3A & B), with only 4.39% and 0.37% of the added LMW-C lost as CO_2 during the 28 day LMW-C addition experiment (Fig. 6). The amounts of SOC-derived CO_2 between soil + BC and "only soil" treatments were similar, but the SOC + LMW-C-derived CO_2 was significantly increased with the addition of biochar (Fig. 3A & B; $p < 0.05$). The addition of LMW-C increased the biochar-derived CO_2 by 11.33% and 23.11% for the Colorado and Oklahoma soils, respectively (Fig. 3C & D).

3.3. Microbial biomass C and dissolved organic C

The soil type, LMW-C, biochar, and their interactions did not change the MBC ($p > 0.05$; Table 4). The MBC ranged across treatments from 13.89 to 16.32 μg C g^{-1} soil and from 11.01 to 17.37 μg C g^{-1} soil for the Colorado and Oklahoma soils, respectively (Fig. 4), and they were almost 50% lower than the MBC values detected in a previous short-term incubation experiment conducted on fresh soils and biochar (Jiang et al., 2016b). The SOC-derived MBC and biochar-derived MBC were also not affected by the soil type, LMW-C, biochar, and their interactions (Tables 5 & 6).

The amount of DOC was significantly affected by the addition of biochar (Table 4; $p < 0.05$), while the soil type, LMW-C, and their

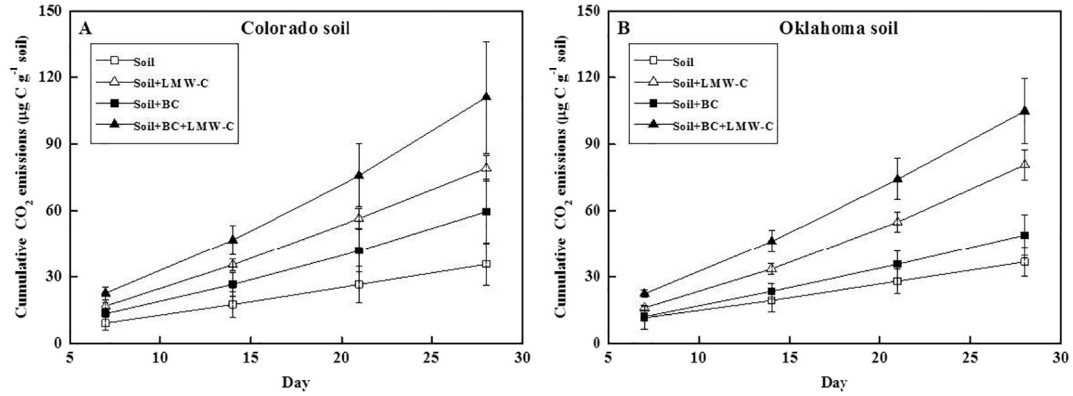

Fig. 2. Cumulative CO_2 emission (μg C g^{-1} soil) with or without the addition of low molecular weight C compounds (LMW-C) and with or without the addition of biochar (BC) over the 28 days of the experiment for A) Colorado soil and B) Oklahoma soil. Error bars show the standard error.

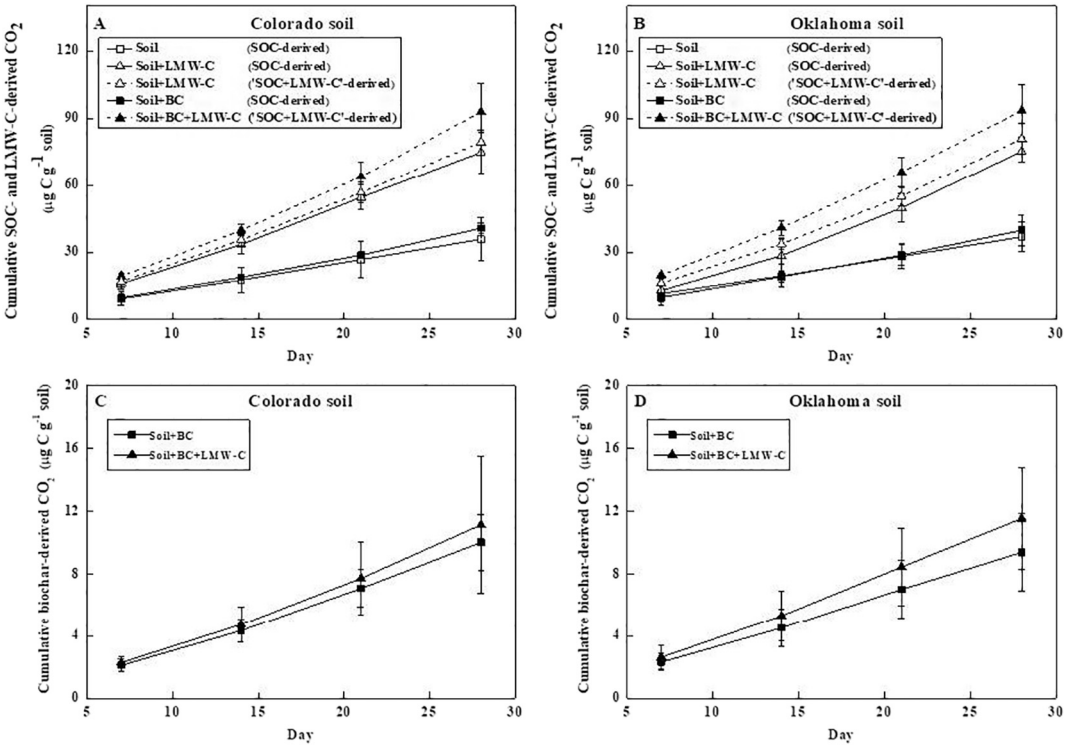

Fig. 3. Cumulative CO_2 emission (μg C g^{-1} soil) derived from different C sources with or without the addition of low molecular weight C compounds (LMW-C) and with or without the addition of biochar (BC) over the 28 days of the experiment. A) Native soil organic C (SOC) and LMW-C-derived CO_2 for the Colorado soil; B) SOC and LMW-C-derived CO_2 for the Oklahoma soil; C) BC-derived CO_2 for Colorado soil; and D) BC-derived CO_2 for Oklahoma soil. Error bars show the standard error.

interactions did not influence it ($p > 0.05$). Interestingly, the addition of biochar increased the DOC concentrations compared to treatments without biochar added. On average, biochar increased the DOC by 39.41% and 99.21% for the Colorado and Oklahoma soils, respectively (Fig. 5). Regardless of the soil type, most of the DOC was derived from native SOC (> 95%), and it was significantly affected by the addition of biochar (Table 5; $p < 0.05$). Only 4.81% and 1.05% of the DOC were derived from the added LMW-C for the Colorado and Oklahoma soils, respectively. On average, 2.82% and 3.48% of the DOC were derived from biochar for the Colorado and Oklahoma soils, respectively.

After 28 days of incubation, we recovered 96.02% and 96.36% of the added LMW-C for the Colorado and Oklahoma soils, respectively. Approximately 78.02% and 92.14% of the added LMW-C were retained in the soil for the Colorado and Oklahoma soils, respectively, with only minor amounts being transferred into CO_2, MBC, or DOC (Fig. 6).

4. Discussion

4.1. Soil organic C decomposition

In this study, we aimed to understand the interactions between the SOC, LMW-C, and aged biochar using aged soils and soil-biochar mixtures. Regardless of the soil type, data on cumulative CO_2 emission suggested that both LMW-C and aged biochar would increase the overall respiration rates (Fig. 2; Table 4), while only LMW-C significantly increased the SOC-derived CO_2 emission (Table 5). Previous studies reported that the addition of LMW-C could increase SOC mineralization through co-metabolism (Mondini et al., 2006; Blagodatskaya and Kuzyakov, 2008). In our study, 34.08% and 16.02% of the MBC were derived from the added LMW-C for the Colorado and Oklahoma soils, respectively (Fig. 4), which was consistent with a co-metabolism mechanism.

Aged biochar did not suppress SOC-derived CO_2 emission in our

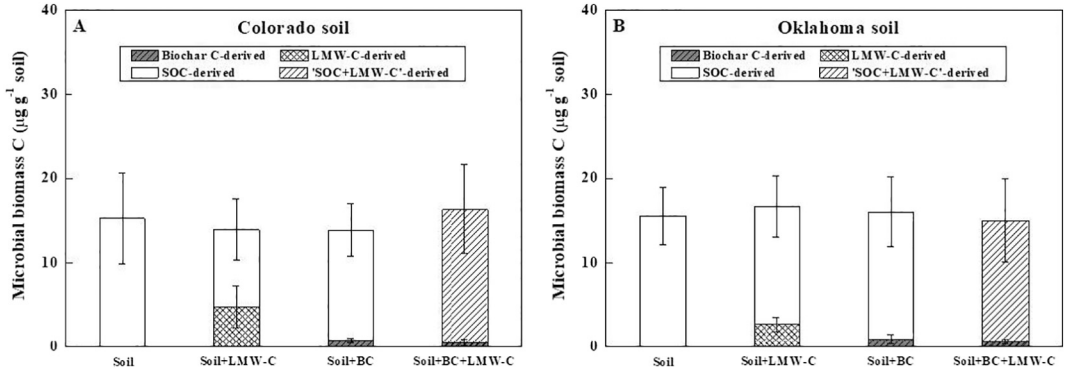

Fig. 4. Microbial biomass C (μg C g^{-1} soil) derived from different C sources with or without the addition of low molecular weight C compounds (LMW-C) and with or without the addition of biochar (BC) after the 28 days of the experiment for A) Colorado soil and B) Oklahoma soil. Error bars show the standard error.

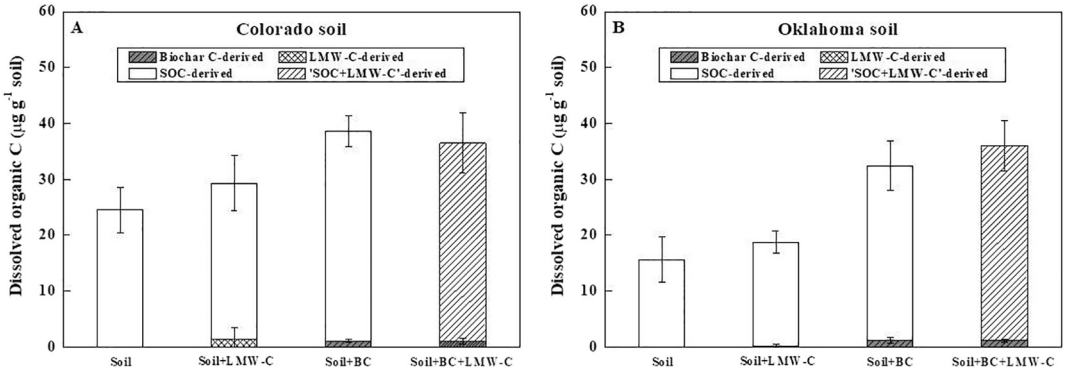

Fig. 5. Dissolved organic C (μg C g^{-1} soil) derived from different C sources with or without the addition of low molecular weight C compounds (LMW-C) and with or without the addition of biochar (BC) after the 28 days of the experiment for A) Colorado soil and B) Oklahoma soil. Error bars show the standard error.

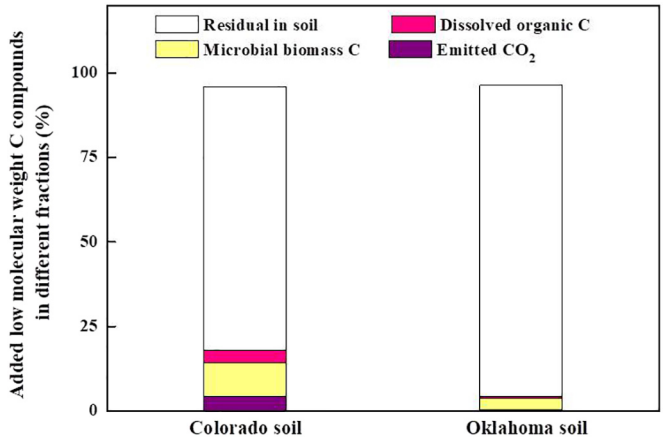

Fig. 6. Recovered low molecular weight C compounds (LMW-C) in different fractions (residual in soil, dissolved organic C, microbial biomass C, and emitted CO_2) of the soil + LMW-C treatment for Colorado soil and Oklahoma soil after the 28 day LMW-C addition experiment.

study, which was in contrast to the negative priming effects reported in previous studies (Weng et al., 2015; Wang et al., 2016). Some previous studies reported that short-term biochar addition promoted soil water contents and increased soil microbial biomass, which in turn increased SOC mineralization (Wardle et al., 2008; Novak et al., 2010), while long-term biochar addition caused negative priming effect on SOC mineralization (Maestrini et al., 2015). Weng et al. (2017) also illustrated that aged biochar could improve soil structure and in turn cause the long-term field negative priming in high clay soil. However in our previous study (same biochar sample was used), we found that the addition of fresh biochar significantly decreased SOC-derived CO_2 emission in the short-term (Jiang et al., 2016b); meanwhile, as the biochar aged in soil, this effect was reversed, and aged char was not as effective for SOC protection compared to fresh biochar. Not only did it promote the CO_2 emission of soil, but it also did not limit the positive priming effect of LMW-C on SOC mineralization (Figs. 3 & 4). One possible explanation of this positive priming effect after 3.5 years from biochar addition may be associated with the high amount of our biochar addition (10% by weight) reducing the bulk density of the biochar-soil system. Previous study has reported that decline in bulk density dramatically increased soil respiration (Torbert and Wood, 1992). While the fresh biochar may well have adsorbed labile C therefore removing it for microbial use causing negative priming in the short term (Jiang et al., 2016b), the change in bulk density and likely higher water content of the soil could well have stimulated microbial activity in the longer term. Our results might be associated with the attenuated SOC sorption capability of aged biochar and decline in N availability of aged soil-biochar mixtures. During the period of biochar aging, the filling or blockage of reactive sites on the surface of aged biochar contributed to the reduction in the sorption capability of the SOC and LMW-C (Oen et al., 2012; Wang et al., 2017), which might cause the decline in SOC stability. Given the initial low surface area of our grass-derived biochar (Table 1), a relatively fast decline in the sorption capacity with time was to be expected. Additionally, our soils had a relatively high sand content (> 50%), thus offered relatively little capacity for mineral-organic associations. Meanwhile, for the biochar addition treatment, the decline in bulk density might have become an important factor influencing SOC mineralization after biochar aging, which promoted SOC loss. On the other hand, in accordance with a previous study (Wang et al., 2015), biochar further intensified the decline in SN availability, as shown by the lower DN in biochar treatments as compared to non-biochar treatments (Fig. 7; Table 4). It can be expected that soil microbial N demand will be further increased in biochar treatment. In our study, the addition of biochar significantly increased the native SN loss of Colorado soil compared to non-biochar treatment during the 3.5 years of aging (Table 3), suggesting that the decrease in N availability caused by the addition of biochar forced soil microbes to decompose native soil organic matter for a N source and might sequentially increase the aged SOC loss. Meanwhile, the addition of LMW-C also significantly decreased the DN in our study (Table 4), suggesting that the microbial utilization of LMW-C would also stimulate microbial N demand.

In our study, the soil DOC was higher in biochar treatments, with almost 95% of the DOC being derived from native SOC, suggesting that the native SOC solubility was increased with aged biochar. This result was consistent with previous studies (Jiang et al., 2016b; Zhang et al., 2017), and an increase in the SOC solubility might be associated with a decline in the sorption capability of aged biochar. An increase in the DOC can promote C loss from the soil system through microbial mineralization and leaching. Our results suggested that the DOC in aged soil-biochar mixtures could be transferred into CO_2 rather than absorbed by aged biochar. This was also consistent with the significant decline in the native SOC of soil + biochar treatments compared to "only soil" treatments observed in the Colorado soil of our study (Fig. 1), which further indicated that the SOC stability might decrease under 3.5 years grass-derived biochar incubation. The inherent biochar recalcitrance is the critical reason why biochar is considered an effective means for SOC sequestration, while the reduction in SOC mineralization by biochar has been claimed as a co-benefit for C sequestration (Singh et al., 2015; Weng et al., 2015). Previous short-term experiments using fresh biochar suggested that the addition of biochar significantly decreased SOC mineralization (Jiang et al., 2016b). However, in this study, results showed that the often claimed co-benefit of biochar reducing SOC mineralization could be lost over time. In the laboratory, the SN was mainly lost through denitrification and the capacity of biochar reducing N_2O emission had been reported in previous studies (Cayuela et al., 2014; Cayuela et al., 2015). However, the persistence of

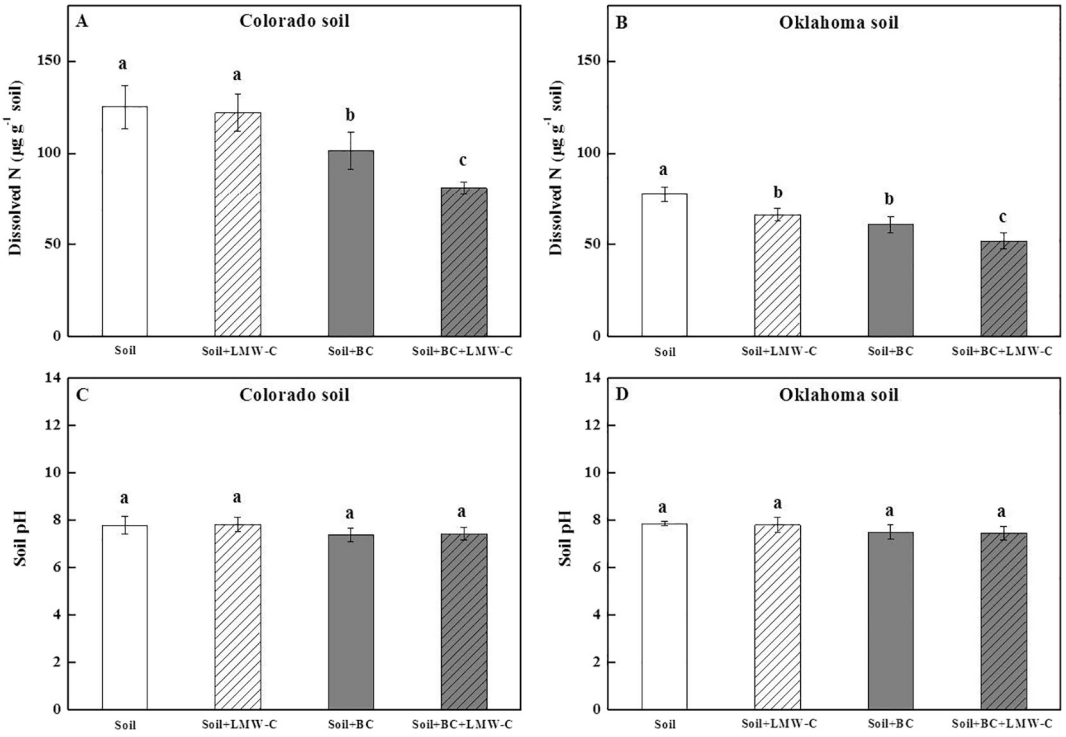

Fig. 7. Dissolved N (μg N g^{-1} soil) and soil pH with or without the addition of low molecular weight C compounds (LMW-C) and with or without the addition of biochar (BC) after the 28 day experiment for A) the Colorado soil and B) the Oklahoma soil. Error bars show the standard error. Different lowercase letters indicate significant differences within each parameter among different treatments.

Table 3
Loss rates of native soil organic C (SOC) and soil N (SN) during 3.5 years of aging. Different letters indicate significant differences within each parameter among different soil types and treatments.

Soil site	Biochar addition treatments (%)	Native SOC (g kg^{-1} soil yr^{-1})	Native SN (g kg^{-1} soil yr^{-1})	Biochar C (g kg^{-1} biochar yr^{-1})
Colorado	0	0.48 ± 0.056 d	0.008 ± 0.007 c	–
	10	0.81 ± 0.081 a	0.028 ± 0.011 b	12.21 ± 1.36 a
Oklahoma	0	0.55 ± 0.017 c	0.068 ± 0.002 a	–
	10	0.65 ± 0.054 b	0.069 ± 0.005 a	3.69 ± 0.28 b

Table 4
Effects of the soil type, low molecular weight C compounds (LMW-C), biochar, and their interactions on CO_2 emission over time, microbial biomass C, dissolved organic C, and dissolved N. Significant effects were indicated using underlined bold *p* values.

	CO_2 emission over time		Microbial biomass C		Dissolved organic C		Dissolved N	
	F	*p*	F	*p*	F	*p*	F	*p*
Soil type	0.440	0.517	0.218	0.647	3.583	0.077	184.987	**< 0.001**
LMW-C	82.722	**< 0.001**	0.313	0.584	0.456	0.509	21.687	**< 0.001**
Biochar	18.533	**< 0.001**	0.019	0.893	16.078	**< 0.001**	56.504	**< 0.001**
Soil type × LMW-C	0.051	0.824	0.478	0.499	0.083	0.777	0.072	0.792
Soil type × Biochar	0.816	0.380	0.000	0.992	0.872	0.364	7.094	**0.017**
LMW-C × Biochar	0.857	0.368	0.380	0.546	0.217	0.648	1.349	0.262
Soil type × LMW-C × Biochar	0.034	0.855	1.155	0.298	0.283	0.602	2.341	0.145

the N_2O suppression effect of aged biochar has been questioned. For example, Spokas (2013) reported that wood biochar lost the initial ability in suppressing N_2O emission after 3 years of aging, which might be due to the loss of biochar adsorbed nitrification/denitrification inhibitors through aging. Our results also suggested that the mechanisms responsible for these alterations in the denitrification depression of aged biochar required further research.

Changes in the microbial biomass, community composition and CUE after long-term incubation may also affect the biochar ability to retain SOC, DOC solubility, and the priming effect on SOC. In our study, the addition of biochar did not increase the soil MBC (Fig. 5) but significantly promoted SOC-derived CO_2 (Fig. 3; $p = 0.021$), suggesting that the microbial CUE might be lower in biochar treatment compared to non-biochar treatment. Soil microbes had been starved for 3.5 years and were adapted to use SOC even under biochar addition. The priming response we observed is a combination of both the aged char with likely saturated surfaces, as well as of a changed microbial community. Microbial composition was not determined in this study, while some previous studies reported that the addition of biochar altered the microbial composition in favor of bacterial groups (Gomez et al., 2014; Hu

Table 5
Effects of the soil type, low molecular weight C compounds (LMW-C), biochar, and their interactions on the soil organic C (SOC)-derived CO_2 over time, cumulative SOC-derived CO_2, SOC-derived dissolved organic C (DOC), and SOC-derived microbial biomass C (MBC). Significant effects were indicated using underlined bold *p* values.

	SOC-derived CO_2 over time		Cumulative SOC-derived CO_2		SOC-derived DOC		SOC-derived MBC	
	F	*p*	F	*p*	F	*p*	F	*p*
Soil type	0.151	0.705	0.000	0.993	3.110	0.103	1.554	0.236
LMW-C	49.508	**< 0.001**	91.416	**< 0.001**	0.339	0.571	0.389	0.544
Biochar	0.193	0.669	0.916	0.357	7.086	**0.021**	0.168	0.689
Soil type × LMW-C	0.693	0.421	0.006	0.939	0.002	0.967	0.220	0.647
Soil type × Biochar	0.103	0.754	0.059	0.812	0.060	0.811	0.617	0.447

Table 6
Effects of the soil type, low molecular weight C compounds (LMW-C), and their interactions on biochar-derived CO_2 over time, cumulative biochar-derived CO_2, biochar-derived dissolved organic C (DOC), and biochar-derived microbial biomass C (MBC).

	Biochar-derived CO_2 over time		Cumulative biochar-derived CO_2		Biochar-derived DOC		Biochar-derived MBC	
	F	*p*	F	*p*	F	*p*	F	*p*
Soil type	1.642	0.236	1.892	0.206	0.339	0.577	0.603	0.460
LMW-C	0.004	0.950	0.013	0.913	0.003	0.959	1.577	0.245
Soil type × LMW-C	0.088	0.774	0.063	0.808	0.003	0.961	0.001	0.972

et al., 2014; Jiang et al., 2016a). It had been reported that bacterial groups could degrade persistent and complex aged SOC (Kramer and Gleixner, 2008), which might promote SOC loss during aging. Meanwhile, the CUEs of bacterial groups were commonly considered lower than those of fungal groups (Keiblinger et al., 2010). A previous study also reported that the addition of biochar could increase the bacterial biomass and in turn cause a decline in the CUE (Jiang et al., 2016a), which might increase the SOC loss.

4.2. Biochar C and LMW-C

In this study, we also sought to investigate the effect of the addition of LMW-C on the stability of aged biochar C. Our results suggested that the stability of aged biochar was not affected by the addition of LMW-C, and the biochar-derived CO_2, MBC, and DOC of the soil + biochar + LMW-C treatment did not show significant differences compared to the soil + biochar treatment (Table 6). Recently, biochar decomposition was observed in laboratory and field experiments through co-metabolism, the enhancement of microbial biomass, or changes in the microbial composition (Kuzyakov et al., 2014; Singh et al., 2014; Jiang et al., 2016a). However, aged biochar, having lost the relatively labile volatile fraction (Dong et al., 2017), may not be as subject to priming.

Interestingly, a large proportion (~85%) of the added LMW-C was retained in the SOC, while ~6%, ~2%, and ~2% of the added LMW-C were recovered from the MBC, DOC, and emitted CO_2, respectively (Fig. 6). The fate of fresh LMW-C was inconsistent with that found in a previous study using the same LMW-C addition rate, in which almost 43% of the added LMW-C was recovered in the MBC 72 h after addition (Strickland et al., 2012). A potential explanation is associated with changes in the MBC after aging. The MBC in aged soil was 50% lower than that in fresh soil samples with fresh biochar (Jiang et al., 2016b), which limited the LMW-C utilization during the 28 days of incubation. Another explanation was associated with the microbial community changes during long-term incubation, which shifted towards organisms with better adaptation in recalcitrant C utilization (Gomez et al., 2014). Thus, most LMW-C was retained in the soil environment rather than being associated or utilized by soil microbes.

Although only a small fraction of the added LMW-C was recovered in soil microbes and emitted CO_2, the addition of LMW-C still promoted aged SOC-derived CO_2 by ~111%, which significantly decreased the aged SOC stability. Old native SOC, which is more persistent and contributes more to long-term C storage than fresh SOC, could thus be affected by a small LMW-C addition even with the addition of biochar.

5. Conclusion

Our study demonstrated that the aged biochar was not as effective for SOC protection as fresh biochar after 3.5 years aging incubation. The decline in SOC stability with aged biochar might be associated with the attenuated sorption of SOC on aged biochar. Decline in attenuated sorption suppressed the limitation of aged biochar on the LMW-C priming effect.

An interesting finding of this study was that the DOC in the aged soil-biochar mixture was significantly higher than that in aged soil, further increasing SOC losses after 3.5 years grass-derived biochar addition to soil. Meanwhile, the addition of LMW-C doubled the aged SOC-derived CO_2. CO_2 emission and DOC leaching were two important SOC loss pathways; therefore, the effect of aged biochar on the soil DOC and the effect of LMW-C on aged SOC stability should be further investigated in field conditions. On the other hand, biochars with contrasting surface areas should be involved in future studies to better evaluate the potential effects of biochar on long-term SOC storage.

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